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(54) **Process for injecting bird embryos into unfertilized receptacle eggs for the production of birds.**

(57) The invention relates to a process for injecting bird embryos into unfertilized receptacle eggs.

It comprises an isolation and an "in vitro" growth of bird embryos obtained by genetic mutation, dilacerations of a fertilized germinative disk, "in vitro" fertilization of oocytes, nuclear transplantation between two embryos, separation and "in vitro" reculture of blastomeres.

Receptacle eggs belonging or not belonging to the species or to the family of the injected embryo.

A device for injecting the embryos that is composed of two needles 5 and 6, which are attached to a device permitting their linear movement relative to each other 11, which is itself fixed to a microhandling arm permitting the collection of embryos from their culture medium and their injection into the receptacle egg yolk 14.

The process according to the invention is intended for the rapid multiplication of poultry or bird strains belonging to rare and/or endangered species.

The present invention relates to Biology and to the production of birds through a process of embryos injection in unfertilized eggs and more precisely, the process brings together the following steps that are: the use of birds embryos, which can be of various origins, for their "in vitro" growth, the injection of one or several embryos per egg that has been carefully chosen and prepared to serve as receptacle matrix, the monitoring of the embryonic development by natural or artificial incubation up until the hatching.

The invention can be applied to the fields of Agriculture for the rapid multiplication of poultry strains obtained by cross-breeding or genetic manipulation, to the fields of Aviculture and of Birds Protection for the rapid multiplication of birds that are rare and/or endangered.

The collections and transfers of embryos have been performed for a very long time in mammals and with the arrival of processes for the "in vitro" fertilization, these are now frequently performed in humans.

The use of such processes in birds faced the fundamental difference in Reproduction Physiology between birds and mammals.

The mature egg of a bird is what is called the egg yolk and the egg possesses within itself all the nutrients that are brought to the mammals through the uterus to ensure the development of the embryo.

This yolk or egg possesses two well distinct parts: The germinative disk that possesses the genetic information, and the yolk itself that corresponds to the cell cytoplasm where the essential elements for the future feeding of the embryo have accumulated. After the ovulation, the egg finds itself, as it travels in the oviduct to lead the bird egg, successively surrounded by albumin and then by a shell. If the egg is not fertilized right after ovulation, the cycle continues in the same way, but the genetic information contained in the germinative disk will have degenerated at the time that the egg is laid.

The object of the current invention is to use these unfertilized eggs that contain all the nutrients needed for the development of the embryo and to very precisely inject them with one or several foreign embryos.

An embryo in its first stage of development is represented by a so-called totipotent cell that possesses the faculty of dividing itself to form, in adequate conditions, a whole and complete individual. In the first stages of the division, the embryo constitutes a solid mass called Morula, which cells are called Blastomeres. The change to the Blastula stage is characterized by the beginning of the shaping of a cavity.

The embryos used in the current invention can be produced: by genetic mutation, by the isolation and culture of totipotent cells collected in the germinative disk of a fertilized egg that has been incubated or not for a few hours, by "in vitro" fertilization of oocytes by spermatozoids, by transplantation of embryonic cells nuclei or of primordial germinal cells into embryonic totipotent cells later cultivated "in vitro", by separation and reculture of Blastomeres up until the Morula or Blastula stage. According to the invention, a large number of embryos, from tens to a few hundreds, of the origin described above, possessing either the same genetic code, or different genetic codes and coming from mutation or not, can be used from within a few hours to a few days.

According to the invention, the embryos are collected under a microscope from the medium that contains them using a micropipette mounted on a microhandling device and each embryo is isolated in the well of a multi-well plate for cell culture, which is then placed in culture between 37 °C and 41 °C until the Morula or Blastula stage is achieved.

The receptacle egg involved in the process of the invention will have particular attributes: It will have to be freshly laid or kept around 15 °C in an aerated area and it can either belong or not belong to the species or to the family that generates the embryo. In the case where the egg would belong to a different species or a different family, it is better to choose the species or family that is phylogenetically the closest to that of the embryo. If this is not possible, the selection of the species providing the receptacle eggs is made according to physicochemical characteristics of the eggs that are the most similar between both species or both families, especially the weight and the volume.

The invention is thus characterized in the fact that it is possible to use eggs belonging to a common species for the production of birds belonging to rare species.

According to the invention, after having been carefully washed, the shell (1) of the receptacle egg is pierced with a hole (2) using a minidrill equipped with a minigrindstone without going through the shell membrane (3). The hole, from 1 mm to 3 mm in diameter, is preferably performed at the equatorial zone level of the egg. A mask with a 5-mm diameter opening is applied to the shell and a solution of liquid plastic is vaporized on the opening. The egg is then allowed to dry in a vertical position, with the largest pole pointing upward.

According to the invention, the injection device is composed of a microhandler, of a set of two needles (2) inserted into one another, and of a microinjector. The needles set is composed of a steel needle (5) with an inside diameter ranging from 0.4 to 1 mm, with a length ranging from 1 cm to 15 cm and with a beveled end; of a glass needle (6) with an inside diameter between 0.2 mm and 0.8 mm and with an external diameter that allows it to slide inside the steel needle. The end exiting from the beveled end of the steel needle is closed and rounded (7). An opening from 0.2 mm to 0.8 mm in diameter is performed on the lateral face of the inferior end of this glass needle (8).

Both needles inserted into each other are fixed at two distinct points (9 and 10) to a device that allows these two needles to undergo a linear movement relative to each other. This device is fixed on the arm of a microhandler (12) such that it can move along all three axes. The glass needle is connected to the microinjector by a polyethylene tube filled with sterile liquid paraffin and the needle itself is filled with a sterile culture medium.

The injection of the eggs according to the invention is performed in the following way: The plate containing the embryos, which are ready to be injected, is placed under the microscope, the microhandler is fixed on one of the sides of the microscope and is equipped with the needles as described earlier. The receptacle egg is placed on a height-adjustable holder, with the largest pole pointing upward, next to the embryos. The opening prepared as described above facing the microhandler.

The end of the glass needle is extended out of the end of the other needle and one or several embryos are sucked in under visual control in a volume of 0.001 mL to 0.02 mL. The glass needle is then inserted back under the protection of the steel beveled needle and the set is positioned to face the opening of the egg (2), according to an angle more or less horizontal. Both needles are then carefully introduced through the shell membrane (3) (Fig. 1), and then through the vitelline membrane (13). The end of the glass needle (6) is then pushed out past the beveled end of the steel needle (5) and the path of both needles continued until the internal face of the vitelline membrane is met. Its location is estimated based on the length of the needles introduced in the egg and on the average diameter of the yolk. A significant push against this membrane will have no serious consequences given that the rounded end of the glass needle (7) will only deform the membrane by touching it, without piercing it.

According to the invention, the embryo or embryos are carefully injected into the egg against the internal face of the vitelline membrane (14) or in any other part of the yolk, in a volume of 0.001 mL to 0.02 mL. The needles are withdrawn following the same initial path and the orifice of the shell (2) is immediately sealed with a drop of silicon putty or of paraffin from a candle liquefied with heat.

The following example illustrates the reduction to practice of the process according to the invention using the germinative disk of a fertilized chicken egg.

The chicken eggs used as receptacle which, according to the invention, will have been selected as described above are carefully washed with water and scrubbed to eliminate any trace of dirt, dried and then disinfected with alcohol. The orifice is prepared as described above. The fertilized egg used is cleaned and sterilized in the same way as the receptacle eggs. It is opened under sterile conditions over a sterile Petri dish and the content is transferred into it. With the help of sterile tweezers, the yolk is moved until the germinative disk is on the top. The disk is then separated from the yolk with sterile scissors and tweezers, and placed in a Petri dish containing the sterile culture medium. The culture medium used herein is TC 199 containing L-glutamine (0.2 mM), inactivated chicken serum (15%), penicillin (100 UI/mL), at pH 7.4 and 280 mosm.

The germinative disk is mechanically dilacerated with the help of sterile needles and the cells are examined with a microscope. The totipotent embryonic cells are recognized by their dimension ranging from 0.021 mm to 0.027 mm in diameter with a very dense and granular cytoplasm. These cells are separated as described above and incubated between 37 °C and 41

°C for 1 hour to 6 hours in a culture oven under 95% air and 5% CO_2. The embryos that have developed are then collected with a micropipette and with the help of a microhandler, grouped by 5 or 6 in a Petri dish containing fresh medium and left at ambient temperature.

One or several embryos are injected in each receptacle eggs as described above and the egg, once closed back, is placed horizontally in an oven between 40 °C and 41 °C, orifice pointing downward, for 6 hours, then in an oven that ensures rotation of the egg at a frequency of 8 to 10 rotations per hour for 18 hours at the same temperature.

After this time, the egg is taken out and can be kept like a classic fertilized egg at 15 °C or placed directly in a classic oven at 37 °C for as long as is necessary for the complete development of the embryo.

CLAIMS

1. A process for the injection of birds embryos in receptacle birds eggs, aimed at the rapid multiplication of poultry strains obtained through cross-breeding or through genetic manipulation, or of birds strains belonging to species that are rare and/or endangered, such process being characterized in that it comprises the following steps:
    a. Isolation and "in vitro" growth of embryos obtained by genetic mutation, by the use of totipotent cells of a fertilized germinative disk, by "in vitro" fertilization of oocytes, by nuclei transplantation between two embryos, by separation and "in vitro" reculture of Blastoderms belonging to an embryo.
    b. Transplantation of the embryos in unfertilized receptacle eggs belonging or not belonging to the species or the family of the injected embryo.
2. The process according to Claim 1, characterized by the fact that the development stage of the embryos involved in the invention ranges from the Morula stage to the Blastula stage.
3. The process according to Claim 1, characterized by the fact that receptacle eggs that belong to a species or a family foreign to the injected embryo have physicochemical characteristics closer to those of the eggs belonging to the species of the embryo, especially with respect to weight and volume.
4. The process according to Claim 1, characterized by the fact that the receptacles eggs used are pierced, before the injection, with an opening in the shell without rupturing the shell membrane (2).
5. The process according to Claim 1, characterized by the fact that the device for the injection of the embryos is composed of two needles (4) controlled by a microhandler, allowing the collection of embryos in their culture medium and their injection in the yolk, in direct proximity to the internal face of the vitelline membrane (14) or at any other location in the yolk of the receptacle egg.
6. The process according to Claims 1 or 5, characterized by the fact that the injection device comprises two needles inserted into one another (4).
7. The process according to Claims 1, 5 or 6, characterized by the fact that the steel needle (5) comprises a beveled end aimed at piercing the membranes.
8. The process according to Claims 1, 5 or 6, characterized by the fact that the glass needle (6) is closed and rounded at its inferior end (7), and possesses at the same end, a lateral orifice (8) through which the embryo can go through.
9. The process according to Claims 1, 5 or 6, characterized by the fact that the end of the glass needle can be either covered or not by the end of the steel needle.
10. The process according to Claims 1, 5 or 6, characterized by the fact that both needles are fixed at two distinct points (9 and 10) on a device, thus ensuring a linear movement of the needles one with respect to the other, wherein the device itself is fixed on the arm of a microhandler (12).